REJECTION UNDER 35 U.S.C. § 112

Claims 3 and 6-12 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification in such a way as to convey to one skilled in the art. This rejection is respectfully traversed.

Applicant respectfully submits that basis can be found for each of the amendments presented throughout the specification, including the examples set forth therein, as originally filed. Thus, no new matter has been introduced.

CONCLUSION

It is believed that all of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider and withdraw all presently outstanding rejections. It is believed that a full and complete response has been made to the outstanding Office Action, and as such, the present application is in condition for allowance. Thus, prompt and favorable consideration of this amendment is respectfully requested. If the Examiner believes that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at (248) 641-1600.

Respectfully submitted,

Dated: October 28, 2002

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β-Glucosidase production by the mycelial culture of the mushroom *Termitomyces clypeatus*

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Extracellular cellobiase activity was detected in the mycelial culture of the mushroom T. clypeatus *with different mono-, di-, and polysaccharides as carbon source. Higher carbohydrate (2–5%) in the medium strongly repressed enzyme production without inhibiting growth rates. On the other hand, nonglucose monosaccharides also could not improve extracellular enzyme activity. Casein hydrolysate (CH) in the medium at 1% (w/v) concentration largely improved enzyme titer irrespective of carbon source (glucose, xylose, cellobiose, starch) used. Extracellular activity also appeared in high carbohydrate media in the presence of casein hydrolysate. The kinetics of extra- and intracellular production of the enzyme in cellobiose (CB) medium, with or without CH, indicated extracellular and growth-dependent production of the enzyme. A maximum intracellular level of 8% of the total cellobiase was measured at the late phase of growth in CB medium. CH had no effect on pH, temperature optima, and thermal stability of the enzyme produced in different carbohydrate-containing media.* T. clypeatus *did not liberate any proteinase in the presence or the absence of CH. Thus CH appeared not to improve enzyme titer by repressing any proteinase or stabilizing enzyme activity liberated in CH-free medium. It was therefore suggested that the constitutive production of cellobiase by* T. clypeatus *was under catabolic repression and CH probably released the repression to some extent.*

The β-glucosidase activity of the culture filtrate on p*-nitrophenyl-β-*D*-glucoside (pNPG), β-methyl-*D*-glucoside, and cellobiose had identical pH and temperature optima at 5°C and 65°C, respectively. The enzyme had higher affinity for aryl-β-*D*-glucoside, while β-CH_3-*D*-glucoside was a very poor substrate for the enzyme. The activity of the enzyme was readily inhibited by glucose, whereas glucose analogues or any other related sugars did not have any appreciable inhibitory activity.*

Keywords: Constitutive cellobiase; mushroom; *Termitomyces clypeatus*; β-glucosidase

Cellobiase, a member of the cellulolytic enzyme complex, has been studied from various cellulolytic bacteria,[1] yeast,[2] and fungi.[3] The enzyme has regulatory functions in the production of exo- and endo β(1 → 4) glucanases[4] and in the *in vitro* saccharification of cellulose.[5] Cellobiase (EC 3.2.1.21) is classified as a specific β(1 → 4) glucosidase that is also active on aryl-β-D-glucoside. Aryl-β-glucosidases without cellobiase activity have also been reported from *Schizophyllum commune*,[6] *Stachybotrys atra*,[7] etc. Cellobiase is supposed to be coinduced with exo- and endocellulases,[8] whereas independent regulation of aryl-β-glucosidases was reported in *Trichoderma viride*[9] and *Neurospora crassa*.[10]

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Received 28 January 1988; revised 28 October 1988

We have reported earlier production and purification of various carbohydrase enzymes[11–14] from the mycelial culture of different mushroom species. In the present communication, we report production of a constitutive and extracellular cellobiase from the mycelial culture of the mushroom *Termitomyces clypeatus*. It should be mentioned that enzymes from mushroom sources have been little studied, although most of the macrofungi are potent lignocellulose degraders.

Materials and methods

Biochemicals

D(+) Cellobiose, D(+) glucose, maltose, β-methyl-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, D(+) glucosamine, L-glucose, D-gluconic acid lactone, D-glucuronic acid lactone, D-gulono-1,4-lactone, D(+) galactose, D(+) xylose, L(+) arabinose, β-methyl

D-xylopyranoside, 2-deoxy D-glucose, dextran (2,34,000), peroxidase (horseradish), glucose oxidase (*Aspergillus niger*), *O*-dianisidine dihydrochloride were purchased from Sigma Chemicals, USA. Starch (soluble) was obtained from Glaxo Laboratories (India) and casein hydrolysate was obtained from Oxoid, Limited (England).

Mushroom

Mycelial culture was developed from the fruiting bodies of the mushroom *Termitomyces clypeatus* (Heim) by the methods as reported earlier.[15]

Mycelial growth of the strain was maintained on enriched agar medium of composition (w/v): glucose 5; malt extract 1; potato extract 10; $CaCl_2 \cdot 2H_2O$ 0.037; KH_2PO_4 0.087; $MgSO_4 \cdot 7H_2O$ 0.05; H_3BO_3 0.057; $CuSO_4 \cdot 5H_2O$ 0.087; $FeSO_4 \cdot 7H_2O$ 0.025; $MnCl_2 \cdot 4H_2O$ 0.0036; $NaMoO_4 \cdot 4H_2O$ 0.0032; $ZnSO_4 \cdot 7H_2O$ 0.03; agar 2; pH 5.

Enzyme production

For propagation in synthetic medium, mycelial growth from the agar medium was initially propagated in the same liquid medium at 28 ± 2°C under shaking. Mycelia obtained after 6 to 7 days of growth were freed from the broth by washing and were blended in 100 ml of sterile water in a Waring blender under aseptic conditions. Two milliliters of the blended suspension was used as inoculum per 100 ml of synthetic medium in a 500-ml flask. Enzyme was produced in medium containing 1% (w/v) of carbohydrate and all the minerals as present in the maintenance medium and 2.463% (w/v) $NH_4H_2PO_4$ was added separately. Mycelia were propagated in shake flasks at 28 ± 2°C. Culture filtrate after growth was dialyzed against 0.1 M acetate buffer, pH 5.0, and used as the source of enzyme.

Enzyme assays

Cellobiase activity was assayed at 40°C using 0.5% (w/v) cellobiose in 0.1 M acetate buffer, pH 5.0. The amount of glucose liberated after 30 min of incubation was estimated by the glucose oxidase-peroxidase method (GOD/POD).[16] The unit of enzyme activity was expressed as micromoles of D-glucose liberated per minute under the assay conditions.

p-Nitrophenyl β-D-glucosidase activity[17] was assayed using 0.45% (w/v) *p*-nitrophenyl β-D-glucoside (pNPG) as substrate in the same buffer. Enzyme activity was determined in terms of micromoles of *p*-nitrophenol released after 10 min incubation at 40°C. Unit of enzyme activity was expressed as micromoles of *p*-nitrophenol released per minute under the assay conditions.

1-0-Methyl β-D-glucosidase activity was assayed in a reaction mixture containing 0.5% (w/v) β-methyl-D-glucoside in 0.1 M acetate buffer, pH 5.0, incubated for 30 min at 40°C. Reducing group was measured by the method of Nelson,[18] modified by Somogyi[19] as described earlier.[12] Unit of enzyme activity was expressed as micromoles of D-glucose equivalent liberated per minute under the assay conditions.

Intracellular cellobiase activity was determined according to Dekker.[20] Mycelia at the various days of growth were washed several times with large volume of ice-cold water and were suspended in minimum volume of 0.1 M acetate buffer, pH 5.0. The mass was then ground in an ice-cold mortar pestle using acid washed sand as abrasive. Breakage of mycelia was confirmed under the microscope. The mixture was then centrifuged at 10,000*g* in cold and the supernatant was dialyzed. The dialyzed sample, either as such or after concentration by lyophilization, was used as the enzyme source.

The proteinase activity of the culture filtrate, whose pH always remained in the acidic range (pH 3–5), was assayed by estimating $OD_{280\ nm}$ of the 5% (w/v) trichloroacetic acid (TCA) soluble fraction of the incubation mixture containing hemoglobin as substrate.[21] The incubation mixture (2.4 ml) contained 2 ml of 2% (w/v) hemoglobin in 0.1 M acetate buffer, pH 5.0, 0.2 ml of dialysed culture filtrate, and 0.2 ml of the same buffer. The reaction was carried out for 30 min at 40°C and was terminated by the addition of 4 ml of ice-cold 5% (w/v) TCA. The mixture was then centrifuged and the optical density of the supernatant was measured at 280 nm.

Characterization of cellobiase activity of the culture filtrate

The optimum pH for the enzyme activity at 40°C was determined using all the substrates (pNPG, cellobiose, and β-methyl-D-glucoside) in buffers of pH range from 1 to 10. The temperature optima for all the substrates in 0.1 M acetate buffer, pH 5.0, were determined within the temperature range of 30°C to 80°C. The effect of addition of different sugars on the enzyme activity was studied using pNPG as substrate. K_m and V_m values for cellobiase, pNPGase, and β-methyl-D-glucosidase activities were determined from the Lineweaver–Burk plot. Protein was estimated according to Lowry *et al.*[22] using bovine serum albumin as standard.

Results and discussion

Production of cellobiase by T. clypeatus *with different carbohydrates as carbon source*

Extracellular enzyme activity in the culture filtrates of mushroom grown in the presence of different carbohydrates (1%, w/v) as carbon source was determined at the ninth day of fermentation when sufficient mycelial growths were observed in all the media. It was observed (*Table 1*) that cellobiase activity was present in all the culture filtrates. However, rates of enzyme production with respect to cellular growth in different monosaccharides, maltose, or starch containing media were more or less similar but were low compared to that observed for cellobiose (CB) medium. The nature of enzyme production appeared like that of inducible

Table 1 Activity of extracellular cellobiase liberated during mycelial growth of *T. Clypeatus* with different sugars (1%, w/v) as carbon source

Carbon source	Mycelial yield (9 days)/(g 50 ml^{-1})	Growth rate (g day^{-1})	Cellobiase activity (U ml^{-1})	U g^{-1} cell mass
D(+) Glucose	0.146	16×10^{-3}	6×10^{-3}	2.095
D(+) Mannose	0.081	9×10^{-3}	2×10^{-3}	1.543
D(+) Xylose	0.101	11×10^{-3}	3×10^{-3}	1.673
Maltose	0.072	8×10^{-3}	3×10^{-3}	2.347
Starch	0.139	15×10^{-3}	4×10^{-3}	1.359
D(+) Cellobiose	0.079	8.77×10^{-3}	62×10^{-3}	38.8
Casein hydrolysate	0.031	3.44×10^{-3}	14×10^{-3}	22.09

The ninth day culture filtrate was dialysed against 0.1 M acetate buffer, pH 5, and was used as the source of enzyme. One-fifth milliliter of 1% cellobiose in 0.1 M acetate buffer, pH 5, was incubated with 0.2 ml of culture filtrate and the reaction was allowed to proceed for 1 h. Then cellobiase activity was measured by GOD-POD method as given in text

enzyme, produced at a basal level in presence of non-inducing carbon sources and being induced by the substrate cellobiose. However, basal synthesis of inducible cellobiase of *T. viride*[23] and of constitutive cellobiase of some *Aspergillus* sp.[4] could not be detected extracellularly unless the intracellular pool or mycelial bound enzyme was searched for.

Stimulation of cellobiase production by casein hydrolyate (CH)

In the determination of activities of different complex nutrients on cellobiase production by *T. clypeatus* in 1% (w/v) CB medium, it was observed that CH, when added at 1% (w/v) concentration, yielded high enzyme activity in the culture filtrate. Enzyme activities in CB media both in the presence and the absence of CH were found to be growth related until the growth optima. But beyond this phase, enzyme activity and cell mass rapidly decreased in both the media. Fall of enzyme activity was similar in CB and CB-CH media, but that of cell mass was relatively faster in CB-CH media.

The kinetics of production of both intra- and extracellular enzyme by the mushroom in the presence or absence of CH in 1% CB-medium were studied (*Figure 1*). The enzyme appeared to be an extracellular one, retaining maximally 5% activity of total in CB-CH medium at the fourth day and 8% at the eighth day in CB medium. However, the intracellular level in CB-CH medium rapidly fell beyond that value, whereas in CB medium the level was retained. The intracellular enzyme level in CB medium was maintained until 12 days of fermentation, when extracellular activity disappeared (*Figure 1*). The rapid fall of the extracellular activity in both the media may be due to progressive inhibition of enzyme activity by the liberated glucose as reported for other cellobiase.[24] Under such a condition, extracellular cellobiase production would not be represented by the measured activities of the culture filtrates. A higher level of intracellular enzyme in CB medium than that in CB-CH medium in the post-optimum growth phase could not be compared unless the actual extracellular levels were known.

It was further observed that casein hydrolysate (1%, w/v) was a poor carbon source for the growth of mushroom and did not support better enzyme activity of the culture filtrate. The rate of enzyme production with respect to the mycelial growth was lower than that found in CB medium (*Table 1*). Thus it appeared that CH could not support higher enzyme activity when present alone and so probably it had some indirect role when present wit other carbohydrates as carbon source.

Effect of CH on cellobiase production in the presence of different carbohydrates

The stimulatory activity of CH did not appear to be specific for the presence of CB as carbon source. Appreciable extracellular activities were also detected when glucose, xylose, or starch was used. The culture filtrate activities in the media containing 1% to 5% (w/v) of these carbohydrates determined at the ninth day of fermentation have been represented (*Figure 2*). The growth rates determined under these conditions (*Figure 3*) indicates regular increase in the mycelial yield with the increase of carbohydrate concentrations. However, enzyme yield with respect to cellular growth was optimum with 1% (w/v) of each of the carbohydrates.

Cellobiase activity in the culture filtrates of mushroom grown in the presence of higher concentrations of glucose, cellobiose, or starch (2–5%, w/v) could not be detected even after 21 days of growth when complete lysis of the mycelia took place. It was not definitely known whether the mushroom was not producting the enzyme or it was being spontaneously inactivated by the product glucose present in higher concentrations. It was also evident (*Figure 3*) that higher concentrations of CB were not repressing enzyme production to such an extent affecting cellobiose utilization for cellular growth. The culture filtrate activity, which was undetectable in these media contain-

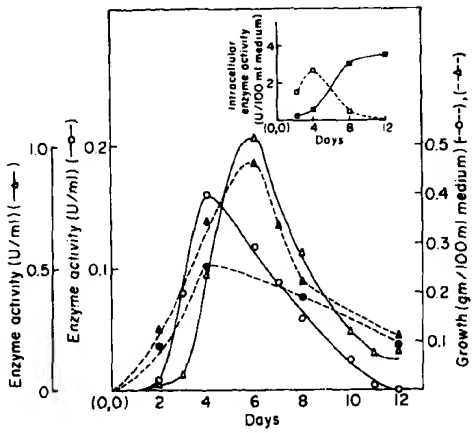

Figure 1 Effect of CH (1%, w/v) on growth, extra-, and intracellular cellobiase production by *T. clypeatus* in 1% (w/v) cellobiose medium. Intracellular enzyme from the mycelia was extracted according to Dekker.[20] The extract and the culture filtrate were dialyzed against 0.1 M acetate buffer, pH 5.0, and were used as the source of enzyme. The cellobiase activity was measured by the GOD-POD method as given in text. Extracellular enzyme in CB-CH (△—△) and in CB (○—○) media; intracellular enzyme in CB-CH (□---□) and in CB (■——■) media; growth in CB-CH (▲—▲) and in CB (●---●) media

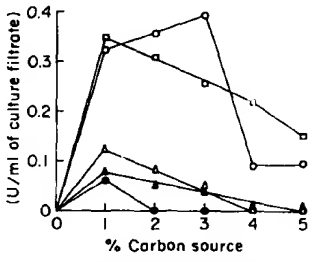

Figure 2 Extracellular cellobiase activity of the mycelial culture of *T. clypeatus* in media containing cellobiose alone or different sugars with casein hydrolysate (1%, w/v). The culture filtrates, dialyzed against 0.1 M acetate buffer, pH 5, were used as the source of enzyme. The cellobiase activity was measured by the GOD-POD method given in text. ●—●, Cellobiose; △—△, glucose with casein hydrolysate; ○—○, cellobiose with casein hydrolysate; □—□, starch with casein hydrolysate; ▲—▲, xylose with casein hydrolysate

ing more than 1% w/v of glucose, starch, or cellobiose, reappeared when casein hydrolysate was supplemented. However, the activity declined slowly with the increase in carbon concentration of the medium. Thus it appeared that production of the constitutive cellobiase by the mushroom was not glucose repressible. Replacement of glucose either by mannose or xylose (*Table 1*) did not improve enzyme production. The addition of CH was found to be effective for the appearance of enzyme activity in the presence of higher concentrations of glucose, starch, or xylose. It was subsequently observed that CH did not have any activity on the *in vitro* physicochemical properties or on the thermal stability of the enzyme liberated in the absence of CH in the medium. Thus it appeared that production of the constitutive enzyme was regulated by catabolic repression as reported for the constitutive β-glucosidase production by *Bacteroides ruminicola*.[25] Addition of CH to the growth media probably was lowering the repression to a certain extent. On the other hand, constitutive intracellular cellobiase of some *Aspergillus* sp.[4] was relatively insensitive to glucose repression and xylose was found to be the best carbon source for the enzyme production.

Properties of the culture filtrate enzyme

Studies on the physicochemical properties of culture filtrate enzyme with respect to its β-glucosidase activity on pNPG, cellobiose, and β-methyl D-glucoside indicated that probably the enzyme was the same. The temperature and pH optima for these substrates were found to be the same at 65°C and pH 5 respectively (*Figures 4* and *5*). It was also observed that enzyme obtained earlier from CB or CB-CH media exhibited identical pH–activity and temperature–activity profiles, and *in vitro* addition of CH (1% w/v) had no effect on them (data not given). The enzyme was reasonably thermostable, losing 15% of its activity after incubation for 2 h at 65°C at pH 5.0, and addition of CH (1% w/v) had no activity on the thermal stability of the enzyme from CB medium. In addition, culture filtrate of CB or CB-CH media had no proteinase activity measured at pH 5.0. Thus stimulation of cellobiase activity by CH was due neither to enzyme stabilization in the culture filtrate nor to repression of any proteinase synthesis by the producer organism which may destabilize enzyme activity in CB medium.

The enzyme was found to be stable between pH 2 and 9. In this context cellobiase from the mushroom appears to be relatively pH stable compared to that reported from *A. niger*,[26] which is one of the potent sources of cellobiase.

The Michaelis constant (K_m) of the enzyme for the substrates pNPG, cellobiose, and β-methyl-D-gluco-

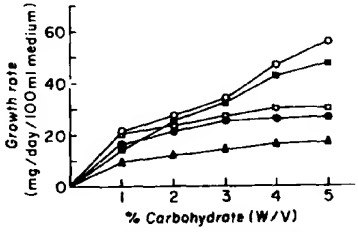

Figure 3 Effect of CH on growth rate in presence of different carbohydrates (1–5%, w/v). Dry mycelial weights were determined at the ninth day of fermentation in media containing varying concentrations of carbohydrate. Growth rate in CB-CH (○—○), G-CH (□—□), CB (●—●), Xy-CH (▲—▲), and St-CH (■—■)

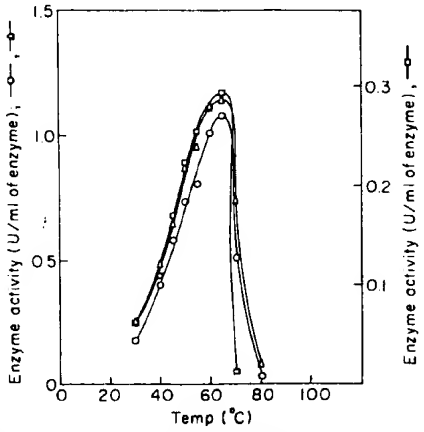

Figure 4 Temperature optima for cellobiase, *p*-nitrophenyl β-D-glucosidase, and 1-O-CH_3-β-D-glucosidase activities. Temperature optima were determined at pH 5 in the temperature range 30°C to 80°C. Methods of assay were described in the text. ○—○, Cellobiase activity; △—△, pNPGase activity; □—□, 1-O-CH_3-β-D-glucosidase activity

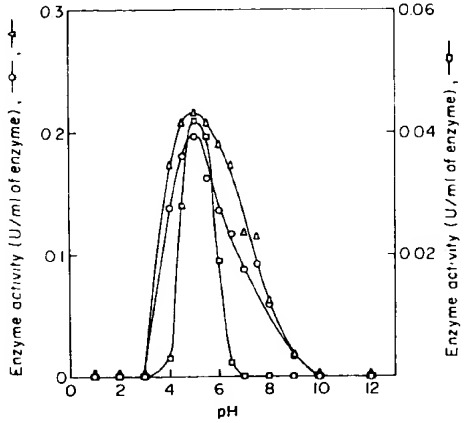

Figure 5 pH optima for cellobiase, pNPGase, and 1-O-CH_3-β-D-glucosidase activities. pH optima were determined at 40°C in the pH range 1 to 10 using different assay methods as described in text. ○—○, Cellobiase activity; △—△, pNPGase activity; □—□, 1-O-CH_3-β-D-glucosidase activity

side were found to be 0.16, 1.105, and 19.36 mM, respectively (*Table 2*). However, the enzyme had very similar V_m values of 0.48 and 0.451 U mg^{-1} of protein for cellobiose and pNPG, respectively, but a very low V_m value of 0.043 U mg^{-1} of protein for β-CH_3-D-glucoside. Thus it is evident that the enzyme had a higher affinity for an aryl-β-D-glucoside, while β-CH_3-D-glucoside is a very poor substrate for the enzyme.

Table 2 Effect of substrate concentration on extracellular cellobiase activity

Substrate	Michaelis constant, K_m (mM)	Maximum reaction velocity, V_m (U mg^{-1} protein)
D(+) Cellobiose	1.105	0.48
p-Nitrophenyl β-D-glucopyranoside	0.16	0.451
β-Methyl-D-glucopyranoside	19.36	0.043

K_m and V_m values were determined by the regression analysis from the slope of the best fitting lines determined from Lineweaver–Burk plot. Activities were determined at 40°C in an incubation mixture containing pNPG (0.06–0.53 mM), cellobiose (0.15–1.5 mM), and β-methyl-D-glucoside (2.6–38 mM) incubated for 30 min with 2.12×10^{-3} U, 7.69×10^{-3}, and 12×10^{-3} U of enzyme, respectively

The *in vitro* inhibitory activity of glucose of various concentrations on β-glucosidase activity using pNPG as substrate is shown in *Figure 6*. The pNPGase activity was found to be relatively sensitive to glucose inhibition. Appreciable inhibition was recorded even in 10 mg ml^{-1} of glucose and only 22.6% activity was observed at 50 mg ml^{-1} concentration. Thus it appears that the enzyme, though produced constitutively, is more sensitive to glucose inhibition compared to the enzyme from some mutants of *T. reesei*[27] or Novozyme 188.[26] Similar to other β-glucosidases, no improvement of enzyme activity was observed on prolonged dialysis of the enzyme which was preincubated with glucose. Thus the absence of enzyme activity in the culture filtrate of *T. clypeatus* grown in the presence of glucose, cellobiose, or starch (2–5% w/v), may be due to the fact that the low amount of cellobiase liberated in the medium was inhibited by glucose. Several attempts (urea, SDS, mild heat treatment) were made to release bound glucose without sacrificing enzyme activity. But none of the attempts was found

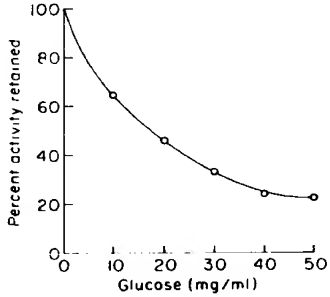

Figure 6 Inhibition of pNPGase activity by glucose. 3.54×10^{-3} U of enzyme was incubated at 40°C for 10 min in 2 ml reaction mixture containing 0.45% pNPG in acetate buffer (pH 5) with different amounts of glucose. Reaction was terminated by 1 cc of 1 M Na_2CO_3. *p*-nitrophenol (pNP) released was measured at 400 nm

Table 3 Inhibitory effect of different sugars and glucose analogues on pNPGase activity

Inhibitor	pNPGase activity (μmol pNP released min^{-1} ml^{-1} of culture filtrate)	% inhibition
None	0.307	0
Glucose (500 μg ml^{-1})	0.103	66.4%
2-deoxy-D-glucose (500 μg ml^{-1})	0.253	17.6%

One-tenth milliliter of the culture filtrate (20 times diluted) was preincubated with 0.1 ml of 0.1% (w/v) inhibitor solution at pH 5 for 15 min. Then 0.25 ml of 0.1% (w/v) pNPG in acetate buffer, pH 5, was added to the incubation mixture and the reaction was carried on for 15 min at 40°C. The enzyme activity was determined according to the assay method as given in the text. Inhibitory activity of different glucose analogues or related sugars like 1-0-CH_3-α-D-glucoside, D(+)-glucosamine, L-glucose, D-gluconic acid lactone, D-glucuronic acid lactone, D(+) galactose, D(+) xylose, L(+) arabinose, β-methyl-D-xylopyranoside, D-gulono-1,4-lactone, dextran (mol. wt. 234,000) was studied under the same conditions. None of them was found to have any inhibitory activity on pNPGase.

fruitful (data not given). It was further observed that the inhibitory activity of glucose on cellobiase activity could be enhanced by preincubation of enzyme with glucose. Preincubation of enzyme with 0.5 mg ml^{-1} of glucose for 15 min inhibited enzyme activity by more than 50%, whereas similar inhibition was achieved when 29 mg ml^{-1} of glucose was added simultaneously with the substrate. This observation further indicated that probably glucose was binding at the same active site of the enzyme for the substrate, causing inhibition. Glucose inhibition was found to be highly specific. Glucose analogues and related sugars, when preincubated with the enzyme at 0.5 mg ml^{-1} concentration for 15 min, did not have any appreciable inhibitory activity on pNPGase. Only 2-deoxy-D-glucose slightly inhibited (17.5%) pNPGase activity when more than 50% inhibition was observed for D-glucose at the same concentration (*Table 3*).

Thus the cellobiase enzyme of the mushroom *T. clypeatus*, though produced constitutively, was regulated by cataboiic repression and the enzyme was sensitive to glucose inhibition.

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